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14. ABSTRACT
The mechanism responsible for the progression of breast cancer to estrogen independence is currently unknown. We have demonstrated a strong correlation between elevated expression of Calgranulin A and Calgranulin B and the loss of estrogen dependence. Experiments using estrogens and antiestrogens have demonstrated that this is not the result of a direct interference with estrogen receptor signaling and must therefore require additional steps. Interestingly, we have also shown that expression of calgranulins in estrogen independent breast cancer cells is quite variable and may be differentially regulated during the cell cycle. Regulation of calgranulin expression by the cytokine Oncostatin M highlights a potential link between the cytokine and estrogen receptor signaling pathways.

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Introduction

The progression of breast cancer from a state of estrogen dependence to estrogen independence is a critical development in the clinical history of this disease. To date, little is known about the crucial changes that lead to the appearance of this more aggressive tumor phenotype which is of importance clinically because it results in resistance to the antiproliferative effects of selective estrogen response modifiers (SERMs), most notably tamoxifen (1-3). In an attempt to understand the changes that allow breast cancer cells to lose estrogen dependence and therefore become resistant to SERMs (4-6),we performed an experiment to identify gene transcripts that were significantly elevated in two unrelated breast cancer cell lines (T47D:C4:2W & MCF-7:2A) that were selected for growth in estrogen-free media, as compared with their estrogen-dependent progenitor cell lines (T47D:A18 & MCF-7:WS8). Using microarray analysis with the Affymetrix U95A gene chip, we identified a number of genes that were greatly elevated in both resistant lines, when compared with their sensitive parental lines. The analysis was designed to focus only on transcripts that were elevated in both lines in order to exclude transcripts that were either spuriously elevated or whose increase was restricted to a single cell line. This was intended to increase the chances of finding genes that were involved in common pathways leading to estrogen independence. Most interestingly, in both pairs of cell lines the two most highly elevated mRNAs were for calgranulin A and its dimerization partner, calgranulin B (7-9) . The finding that the transcripts for two related gene products were elevated more than 30 fold in the case of calgranulin B and more than 10 fold for calgranulin A, in two independently derived cell lines, strongly suggested a mechanistic pathway that may be responsible for the loss of estrogen dependence in these breast cancer cell lines. This may represent a widespread mechanism by which cells can progress to a more aggressive phenotype accompanied by a loss of estrogen dependence.

Body

Experiments were undertaken to demonstrate a correlation between calgranulin expression and lack of estrogen dependence in breast cancer cell lines. A panel of breast cancer cells, including lines derived from common parental lines (e.g. [MCF-7:WS8, MCF-7:2A and MCF-7:C4-12-5]; [T47D:A18 and T47D:C4:2W]) as well as unrelated breast cancer lines (MDA-MB-231, ZR-75-1 and MDA-MB-468) were run in a standard western blot and probed for calgranulin A (Cal A) using the 85C2 monoclonal antibody. The blot was then reprobed with an antibody to estrogen receptor alpha (ER-α). The blot was finally probed with α-tubulin to confirm equal loading.

The data in Figure 1 and Table 1 show a significant correlation between loss of estrogen dependence and expression of Cal A. This suggests that expression of Cal A may be either an indicator of the propensity of cells to lose estrogen dependence or a result of the loss of estrogen dependence. The only cell line that does not fit this model is the MDA-231 cell line, which expresses neither ER-α nor Cal A.

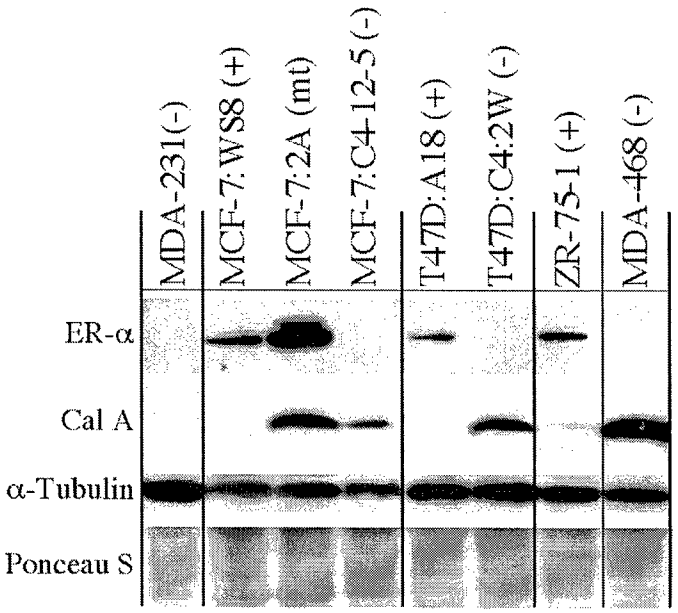

Figure 1: Expression of calgranulin A in breast cancer cell lines. Whole cell extracts were prepared from log phase cells and run in a standard western blot. The blot was first probed with a monoclonal antibody to Calgranulin A (Cal A), reprobed with an antibody to estrogen receptor alpha (ER-α) and finally probed with an antibody to α-tubulin as a loading control. Ponceau S staining of the membranes is included as a second control for loading.

Table 1: Cell Line Characteristics

Cell Line	ER Status	Estrogen dependence	Cal A Expression
MDA-231	-	-	-
MCF-7:WS8	++++	+++	-
MCF-7:2A	++++(mutant)	-	+++
MCF-7:C4-12-5	-	-	++
T47D:A18	+++	++++	-
T47D:C4:2W	-	-	+++
ZR-75-1	+++	+++	+
MDA-468	-	-	++++

To address the possibility that Cal A expression may be a direct result of inhibition of ER-α signaling, WS8 and A18 cells were grown in estrogen free media for 4 days to remove all endogenous steroids, replated and then treated with 1nM 17ß-estradiol (pure estrogen), 100nM 4-hydroxytamoxifen (SERM) or 100nM ICI 182,780 (pure antiestrogen) for an additional 2 days. Whole cells lysates were then prepared and run in a standard western blot. ER-α expression serves as a control for the effects of the steroids used. Estradiol has been shown to cause a downregulation of ER-α, 4-OHT has been shown to stabilize ER-α and ICI 182,780 has been shown to cause a rapid degradation of ER-α in MCF-7:WS8 cells. The response is similar in T47D cells with the exception that estradiol does not cause a downregulation of ER-α (5). In both the WS8 and A18 cell lines, Cal A remained below detectable levels in all treatment groups. Control lysates from MCF-7:C4-12-5 and T47DC4:2W cells were included as positive controls for Cal A expression. This confirms that Cal A expression is not likely to be a direct downstream target of estrogen receptor signaling and probably requires other pathways to become overexpressed.

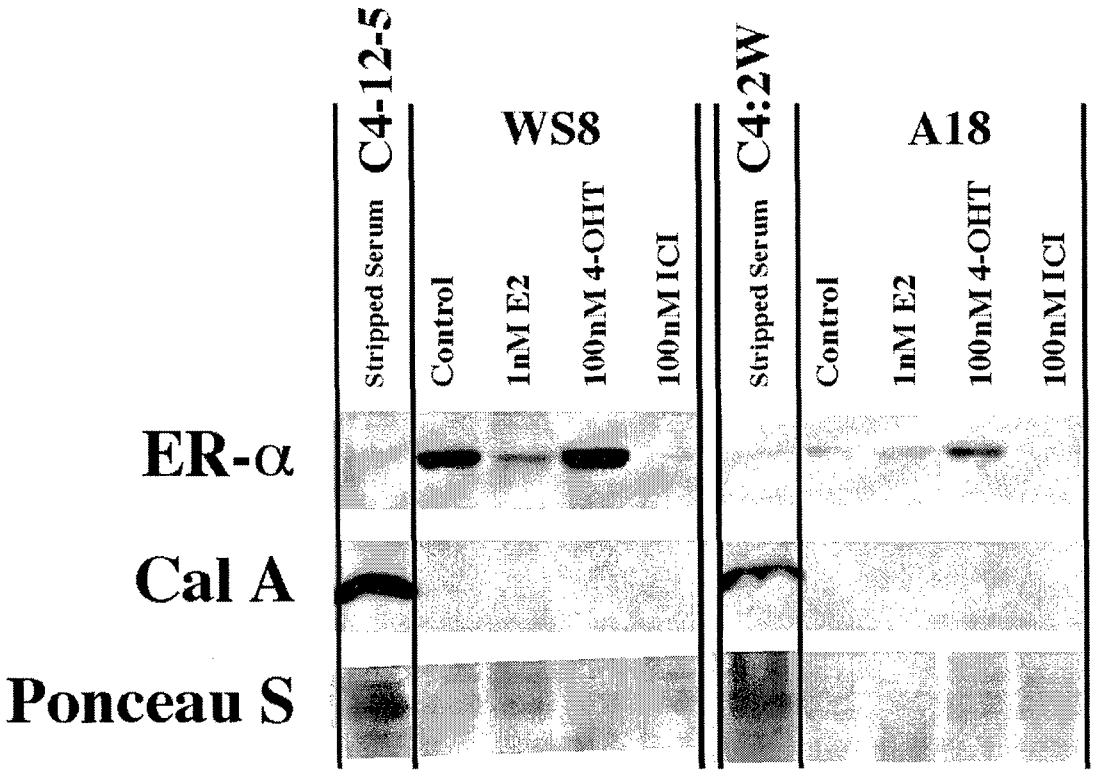

Figure 2: Estrogen/antiestrogen regulation of Cal A expression. MCF-7:WS8 and T47D:A18 cells were grown in estrogen-free media for 4 days, replated and treated with the indicated drugs for an additional 2 days. Whole cell extracts were prepared and run in a standard western. Cal A and ER-α expression were determined as described above.

To generate cell lines which mimic the Cal A expression in the MCF-7 and T47D background, MCF-7WS8 and T47D:A18 cells were transfected with a construct received from Karen Ross and Mark Herzberg (Univ. of Minnesota) which included the cDNAs for Cal A and Cal B separated by an IRES. Pooled populations were isolated and screened for Cal A and Cal B expression (data not shown). Cal A expression was reasonable, however, Cal B expression was quite low. It was subsequently revealed by the manufacturer of the vector (Clontech Biotech) that the IRES included in the original construct used to generate this vector is attenuated and regularly gives rise to low or undetectable expression of the downstream cDNA. Unfortunately this information was forwarded only after the cell lines were generated and initially screened. This is consistent with our observations and

therefore these cells lines are of limited use in these studies. Attempts to transfect the cells with expression vectors from another source were unsuccessful.

We then employed a cell sorting approach to enrich our cell population with calgranulin expressing cells. EGFP is a co-expressed gene on another set of Cal A and Cal B expression vectors received from Ross and Herzberg (10). MCF-7:WS8 and T47D:A18 cells were transfected with Cal A, Cal B or a mixture of Cal A and Cal B vectors. All cells were also co-transfected with pCDNA3, to provide a drug selectable marker (G418 resistance). Cells were selected in G418 until a stable population grew out. These cells were then sorted for EGFP expression in a Beckman Coulter EPICS Elite flow cytometer, using a 488nm air-cooled laser. For both MCF-7:WS8 and T47D:A18 transfectants, less than 5% of the cells were positive for EGFP during the first sort. The small number of EGFP positive cells forced us to perform a second round of sorting, in which 25-40% of the cells were EGFP positive. After these EGFP positive cells grew out they were subjected to western analysis. Technical difficulties with the Cal B antibody have prevented us from analyzing the expression of Cal B in the cells thus far. However, Cal A expression in the Cal A alone and Cal A + Cal B

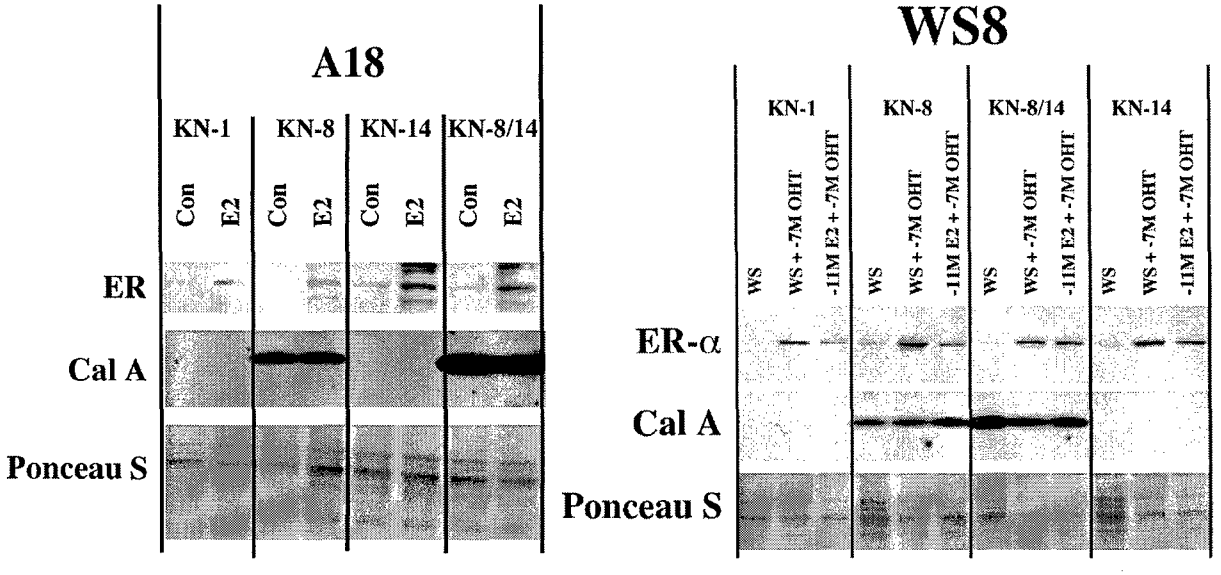

Figure 3: Expression and hormone regulation of Cal A and Cal B after transfection. MCF-7:WS8 (WS8) and T47D:A18 (A18) were transfected with expression plasmids for Cal A (KN-8) and/or Cal B (KN-14), or empty vector (KN-1) all of which included an EGFP cDNA. After two rounds of cell sorting, cells were treated with 17ß-estradiol (E2), 4-hydroxytamoxifen (OHT) or both for 48 hours as shown. Blots were probed for Cal A and subsequently reprobed with Estrogen Receptor-α (ER) in a standard western blot. Ponceau S is included to demonstrate equivalent protein loading.

transfectants has been robust as shown in Figure 3.

The role of estrogen in regulation of exogenous Cal A expression was then assessed in the T47D:A18 cells. Cells were deprived of estrogen for 4 days, and then 1nM 17ß-estradiol was added to the growth media for 48 hours. Cell lysates were prepared and run in a standard western blot. As shown in figure 3, estradiol had no significant effect on the expression of Cal A in these cells. Cells with exogenous expression of Cal showed no change in expression and control cells showed no measurable expression under either condition, as had been previously observed. Similar results were obtained with the MCF-7:WS8 cells (data not shown). In another experiment MCF-7:WS8 cells were grown in control media containing whole serum, alone (WS), or supplemented with 100nM 4-hydroxytamoxifen. In addition, another group of cells were grown in media containing charcoal stripped serum supplemented with 0.01nM 17ß-estradiol in combination with 100nM 4-hydroxytamoxifen. In all transfectants expressing Cal A, hormonal treatment had no significant effect on Cal A expression. Importantly, Cal A expression had no effect on the regulation of ER expression after the various hormonal treatments in either cell line.

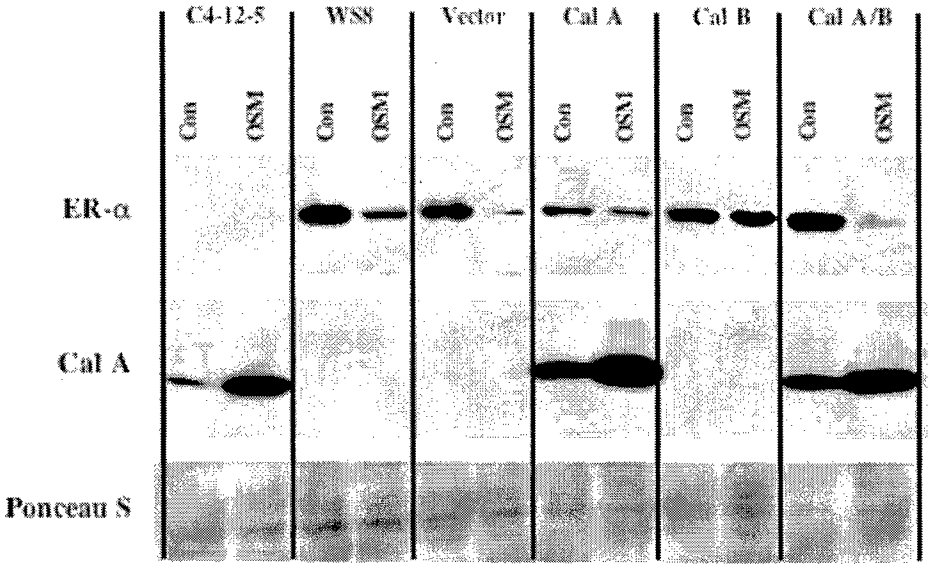

Figure 4: Effect of Oncostatin M on Calgranulin A Expression. Cells were treated with 25ng/ml Oncostatin M in media containing 2% fetal bovine serum for 24 hours. Western blot was then performed as described above. Ponceau S is included to demonstrate equivalent protein loading.

A recent report demonstrated that the cytokine Oncostatin M (OSM), a member of the IL-6 family of cytokines, could significantly stimulate the expression of calgranulins in MCF-7 cells. Interestingly, this was accompanied by significant growth suppression (11). We repeated these studies in our control and calgranulin transfected cells. As shown in Figure 4, exposure of MCF-7 cells to 25ng/ml OSM for 24 hours caused a very significant increase in Cal A levels. Interestingly Cal A expression was not observed in the parental or vector alone cells, but the transfected cells showed an elevation in Cal A levels. The ER negative C4-12-5 cells also showed an increase in Cal A expression after treatment with OSM. This observation suggests that the effect of OSM on Cal A expression is on the stability of the protein and may not be a transcriptional effect. This observation will be investigated in future studies.

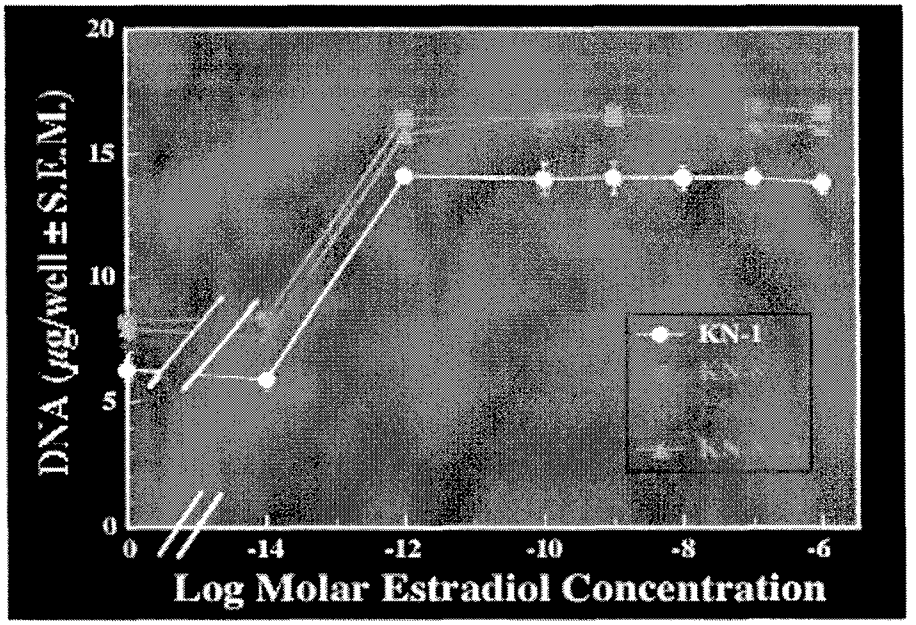

Figure 5. Effect of Calgranulin Expression on Estrogen Dependant Growth. Cells were grown in estrogen depleted media for 4 days, plated in 48 well dishes and treated the following day, in triplicate, with a log dose range of 17ß-estradiol (0 to 1μM). Cells were allowed to grow for 6 days and DNA content was measured using Hoescht 33258 dye fluorescence as a measure of cell growth.

The effect of calgranulin expression on estrogen dependent growth in MCF-7:WS8 cells was tested in a standard 6-day growth assay. As shown in Figure 5, expression of calgranulin alone is not sufficient to alter the dependence of MCF-7:WS8 cells on estrogens. A similar effect was seen in T47D:A18 cells (data not shown).

Key Research Accomplishments

- ❖ Established correlation between estrogen independence and calgranulin overexpression in established breast cancer cell lines
- ❖ Initial observation of differential calgranulin expression in log phase cells
- ❖ Generated shRNA vector specific for calgranulin A
- ❖ Demonstrated cytokine regulation of calgranulins in breast cancer cells
- ❖ Generated cell lines expressing Cal A, Cal B or both.

Reportable Outcomes

Preliminary data generated from this award were included in a Research Scholar Grant proposal to the American Cancer Society, which received the highest score in the Clinical Cancer Research and Epidemiology Committee (first out of 38 applications). Final notice of award is currently pending.

Findings were presented in a poster session and an invited talk at the ERA of Hope Meeting in Philadelphia, PA in June of 2005.

MCF-7 and T47D cell lines expressing Cal A and Cal B have been generated.

Conclusions

Data generated during these studies, in combination with clinical studies funded by Pathology Associates of University Hospitals, has established a very intriguing link between calgranulin expression and estrogen receptor signaling in breast cancer cells. The work presented here support more extensive studies investigating this pathway. In addition to the signaling component, the potential utility of calgranulin expression as prognostic biomarkers could prove very useful in the clinical treatment of breast cancer.

The demonstration of cytokine regulation of steady state protein levels of Cal A suggests a possible interaction between these pathways. The finding that calgranulin expression alone is insufficient to significantly alter growth response to estrogens suggests that other alterations are required, or calgranulin expression is a downstream indicator of the loss of estrogen responsiveness in breast cancer cell lines. Future studies will address these questions and others regarding the role of calgranulin expression in breast cancer progression.

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